Supporting Online Material for

Inter-conversion Between Intestinal Stem Cell Populations in Distinct Niches

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Materials and Methods

Generation of *Hopx*$^{ERCre/+}$ mice

Hopx$^{ERCre/+}$ mice were generated by homologous recombination in the embryonic stem cells targeting an *IRES-ERCre-Frt-PGK-neo-Frt* cassette to the 3' untranslated region of the endogenous *Hopx* locus. The *PGK-neo* cassette was removed by breeding initial progeny to mice expressing ubiquitous FlpE recombinase (*ACTFLPe* (*25*)).

Mice

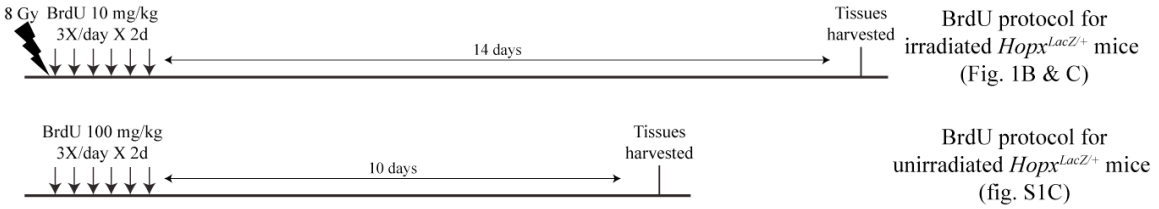
Hopx$^{LacZ/+}$ (*12*), *R26*$^{LacZ/+}$ (*17*), *R26*$^{mT\text{-}mG/+}$ (*26*), and *R26*$^{Tom/+}$ (*27*) mice have been described previously. All mice were maintained on mixed genetic backgrounds. The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal protocols.

Tamoxifen induction

Mice of at least 50 days old were injected intraperitoneally with tamoxifen (100mg/kg body weight; Sigma) dissolved in corn oil, as a single dose or daily for 5 consecutive days, as indicated.

BrdU retaining assay

For labeling during gut development, *Hopx*$^{LacZ/+}$ mice (aged 5 weeks) were injected intraperitoneally with BrdU (100mg/kg body weight; Sigma) three times daily for 2 days. Tissues were collected 10 days after BrdU administration. For labeling during post-irradiation crypt regeneration, *Hopx*$^{LacZ/+}$ mice were irradiated (aged 6-8 weeks; 8 Gy) and then injected intraperitoneally with BrdU (10mg/kg body weight) three times daily for 2 days (*15*). Tissues were collected 14 days after BrdU administration. Tissue sections were then stained with X-gal and an anti-BrdU monoclonal antibody (1:50 dilution, HybridomaBank). Non-Paneth BrdU-retaining cells per circumference section of gut were quantified: 0.63 on day 10 (38 retaining cells per 60 circumferences) in the unirradiated samples and 3.3 on day 14 (79 retaining cells per 24 circumferences) in irradiated samples, similar to results previously reported (*15*).



LacZ staining

Tissues were fixed for 2 hours in ice-cold fixative (1% formaldehyde, 0.2% gluteraldehyde and 0.02% NP40 in PBS) (*5*) at 4°C, rinsed twice in PBS for 20 minutes at room temperature, and then incubated for 12 hours at room temperature in the staining solution [2mM $MgCl_2$, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 0.01% NP40, 0.01% sodium deoxycholate and 1mg/ml X-gal in PBS]. Detergents were removed from the staining solution for cultured organoids. The stained tissues were fixed in 2% paraformaldehyde, ethanol dehydrated, and sectioned using standard methods.

Crypt isolation, cell disaggregation and culture

Crypt isolation, cell dissociation and culture were performed, as described previously (*24*) with minor modifications. Isolated small intestines were exposed longitudinally and washed with cold PBS. Villi were removed using a glass coverslip. The tissue was chopped into approximately 5 mm pieces, washed with cold PBS, and incubated in 5 mM EDTA in PBS for 5 minutes at room temperature. The tissue fragments were suspended vigorously with a 25-ml pipette in cold PBS. The supernatant, mostly villi, was discarded. The sediment was resuspended with 5 mM EDTA in PBS and incubated for 45 min on ice. The supernatant was replaced by fresh PBS and resuspended vigorously, yielding supernatants enriched in crypts. Crypt fractions were immediately diluted (1:1) with advanced DMEM/F12 (Invitrogen) containing Glutamax (Invitrogen) (ADF medium) and centrifuged at 1,200 rpm for 3 min at 4°C. The crypt pellets were resuspended in 10 ml ADF, and samples were passed through 100 μm and 70 μm filters (BD Biosciences) to remove residual villous material. The crypts were centrifuged at 600 rpm for 3 minutes at 4°C and single cells were discarded. The sediment was purified crypts and used for culture or single cell disaggregation. For crypt disaggregation into single cells, the purified crypt pellet was resuspended in SMEM (Invitrogen) containing 1 mg/ml Trypsin (Calbiochem), 0.8 KU/ml DNase (Sigma) and 10 μM ROCK pathway inhibitor Y-27632 (Sigma), and cultured for 30 minutes at 37°C with occasional re-suspension. After disaggregation, cells were sequentially passed through 40 μm cell strainers (BD Biosciences) and 20 μm mesh filters (Millipore).

Crypts were embedded in Matrigel (BD biosciences) at 1,000 crypts/ml, and 30 μl of Matrigel was seeded on pre-warmed 48-well plate. After the Matrigel solidified, advanced DMEM/F12 medium (Invitrogen) containing 50 ng/ml EGF (Invitrogen), 100 ng/ml Noggin (PeproTech), 1 μg/ml R-spondin1 (R&D systems), supplemented with 10 mM HEPES (Sigma), Glutamax (Invitrogen), N2 supplement (Invitrogen), B27 supplement (Invitrogen) and penicillin/streptomycin (Invitrogen) was overlaid. Growth factors were added every other day and the entire medium was changed every 4 days. To induce Cre recombinase activity in organoids, tamoxifen was added for 12 hours on day 0.5. Single cells were analyzed using BD FACS Caliber and DiVa cell sorter (BD biosciences). Cells were stained with anti-CD45-APC (eBioscience) to exclude all hematopoietic cells. 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide were used to exclude dead cells. Sorted cells were collected and embedded in Matrigel containing 1 μM Jag-1 (AnaSpec), followed by seeding on a 96-well plate (100 single cells; 10 μl Matrigel per well). Crypt culture medium containing 10 μM Y-27632 (Sigma) and 1 μM *N*-acetylcysteine (Sigma) was overlaid. 100 ng/ml Wnt3A (R&D systems) was added in the culture medium as indicated for up to 4 days. Cells were fixed and stained with DAPI to visualize cell nuclei to count cells per organoid. DAPI stained nuclei were quantified.

In order to detect LacZ and tdTomato expression of $Hopx^{LacZ/+}$;$Lgr5^{EGFP\text{-}ERCre/+}$;$R26^{Tom/+}$ mice, a purified crypt pellet was prepared and resuspended in SMEM (Invitrogen) containing 0.2 % collagenase type 3 (Worthington Biochemical), 0.8 KU/ml DNase

(Sigma) and 10 μM ROCK pathway inhibitor Y-27632 (Sigma) into a near single cell suspension, and cultured for 20 minutes at 37°C with occasional re-suspension. After disaggregation, cells were fixed for 10 min in an ice-cold fixative, and then stained with X-gal for 2 hours as described elsewhere. Cell suspensions were coverslipped with Fluoromount (Sigma). The mice were induced on the same day, and cell suspensions were prepared on the days indicated.

RNA extraction and real-time PCR

Quantitative real time PCR was performed as described elsewhere (*28*). Briefly, cells were FACS-sorted directly into Trizol LS (Invitrogen) and total RNA was extracted using the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed using SuperScript First-Strand Synthesis Kit (Invitrogen). Real-time PCR was performed using Power SYBR Green PCR Master Mix on the StepOnePlus Real-Time PCR System (Applied Biosystems). For RNA isolation from formalin-fixed paraffin-embedded (FFPE) intestines, intestines were briefly fixed and stained with X-gal as described elsewhere for 3 hours. The stained tissues were fixed in 2% paraformaldehyde, ethanol dehydrated, embedded in paraffin and sectioned using standard methods. RNA was extracted from X-gal stained intestines using PALM (Zeiss) microdissector and RNeasy FFPE kit (Qiagen). PCR conditions and primer sequences are available upon request.

Histological analysis

Tissues were fixed in 2% paraformaldehyde, ethanol dehydrated, embedded in paraffin, and sectioned as described previously (*29*). Eosin and periodic acid-Schiff (PAS) staining were performed using standard methods. We used primary antibodies to GFP (1:500 dilution, Abcam), and chromogranin A (1:1000 dilution, ImmunoStar). Bmi1 immunohistochemistry (1:100 dilution, Bethyl Laboratories) was performed using X-gal-stained frozen sections. Antibody specificity was assessed using a specific blocking peptide (5 μg blocking peptide/μl of antibody, Bethyl Laboratories). Bmi1 images (fig S7A, B, and D) were taken on the same microscope, using the same camera and light settings. All immunohistochemistry was visualized on the Nikon Eclipse 80i fluorescence microscope, except the confocal images which were evaluated on the Leica CTR6000. All images were analyzed using Adobe Photoshop (sizing, brightness or contrast adjustments, etc). Brightness and contrast was adjusted linearly across the entire image for any particular image.

Statistical Analysis

Data are shown as mean ± 1 s.d. Comparisons between multiple groups were made using 1-way ANOVA followed by a post-hoc Bonferroni correction, $P<0.05/n$ for significance.

Supporting References

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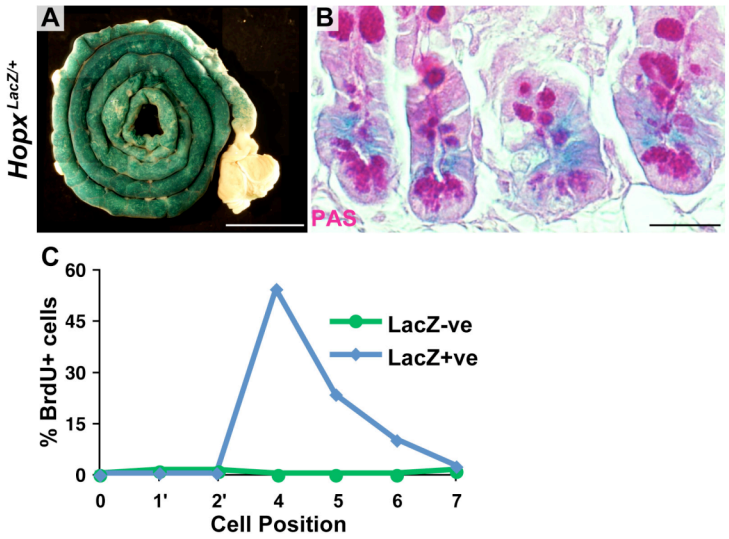
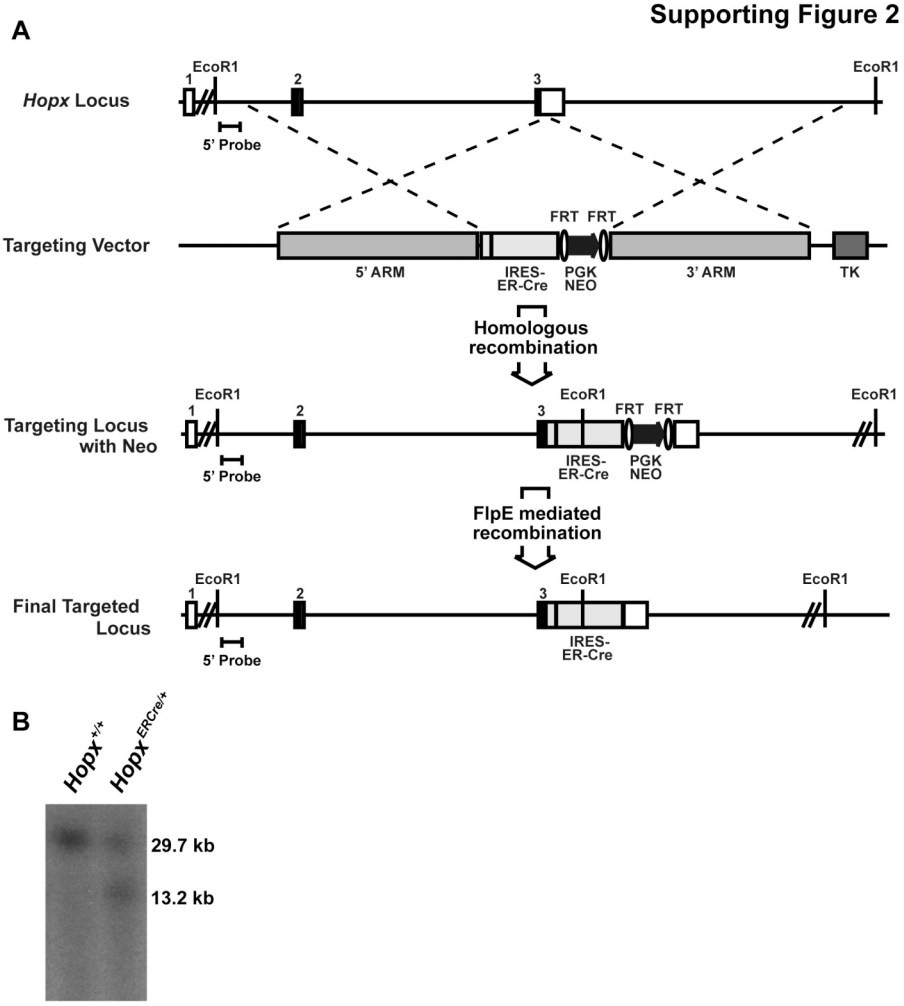
Supporting Figure 1



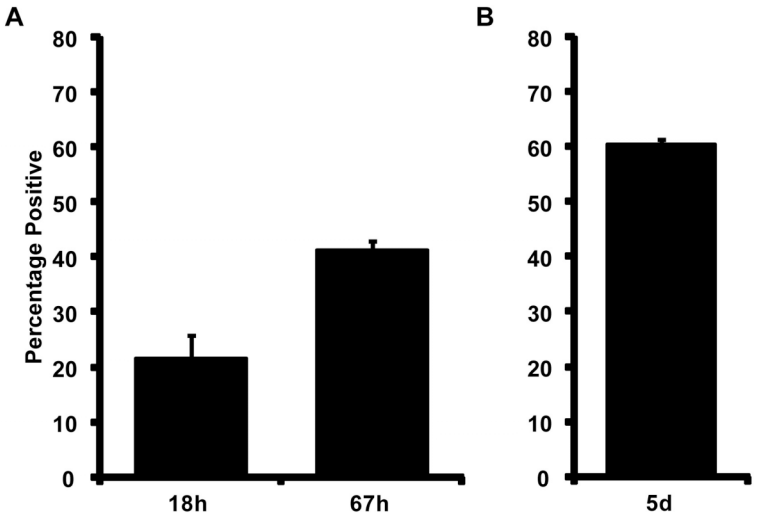
Supporting Figure 1: *Hopx* expression
(A) *Hopx* is expressed across the length of the small intestine. (Composite image, 9 images combined into 1) **(B)** *Hopx* is expressed in the +4 position, above the PAS+ Paneth cells. **(C)** Quantification of BrdU positive cells at each crypt position in unirradiated $Hopx^{LacZ/+}$ mice. Scale bars: 25 μm (B) and 1000 μm (A).

Supporting Figure 2



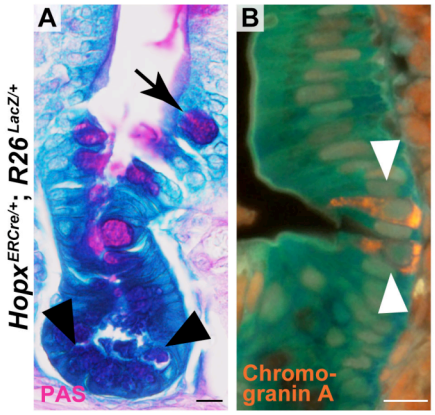
Supporting Figure 2: Generation of *Hopx*$^{ERCre/+}$ mice
(A) Schematic of the knock-in approach in which *IRES-ERCre* was targeted to the 3' untranslated region of *Hopx*. *PGK-neo* was excised by FlpE-mediated recombination. **(B)** Southern blot analysis of wild-type (WT) and heterozygous knock-in (KI) mice. Genomic DNA was digested with EcoRI and subjected to hybridization with the probe indicated in (A). The WT and KI alleles produced 29.7 kb and 13.2 kb bands, respectively.

Supporting Figure 3



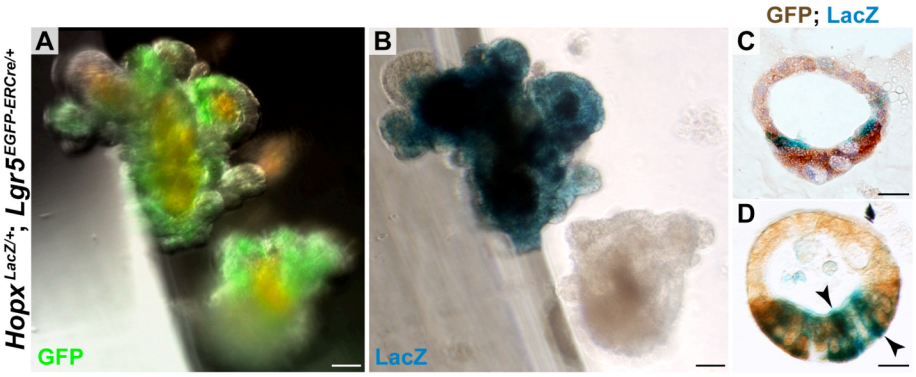
Supporting Figure 3: Labeling efficacy of *Hopx*$^{ERCre/+}$;*R26*$^{LacZ/+}$ mice
Percentage of X-gal-stained crypts relative to the total number of crypts in sections of the proximal intestine quantified (n=3 mice for each time point). Analysis performed 18 hours or 67 hours (372 and 320 crypts counted, respectively) after a single pulse of tamoxifen (A) and 1 day after the last day of a 5 day tamoxifen pulse (377 crypts counted, B).

Supporting Figure 4



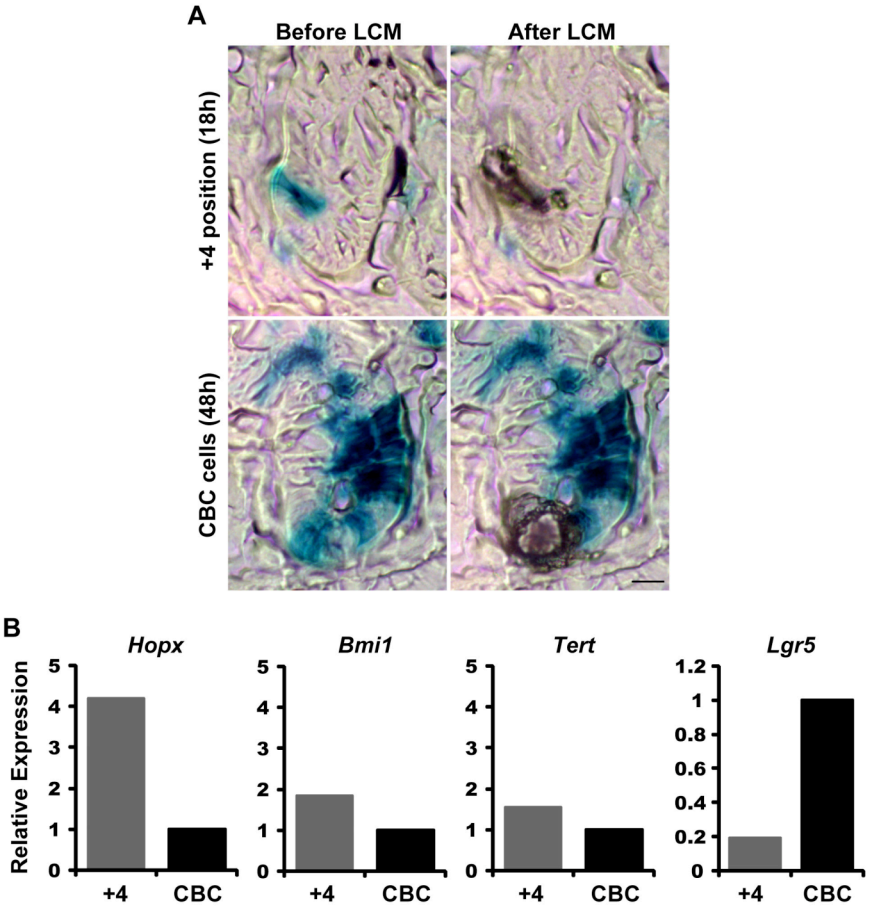
Supporting Figure 4: *Hopx* cells give rise to all intestinal epithelial derivatives
(A) *Hopx* expressing cells give rise to Paneth cells (arrowhead) and goblet cells (arrow), both PAS positive. **(B)** *Hopx* expressing cells give rise to chromogranin A positive neuroendocrine cells (X-gal stained section). Analysis was performed 2 months after a 5 day tamoxifen pulse. Scale bars: 10 μm (A and B).

Supporting Figure 5



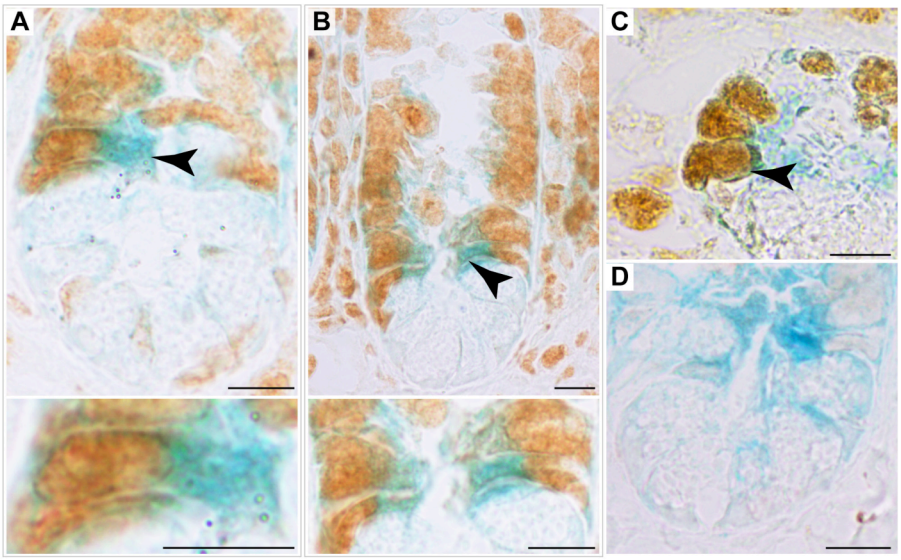
Supporting Figure 5: Organoid cultures from *Hopx*$^{LacZ/+}$;*Lgr5*$^{EGFP-ERCre/+}$ mice
(A and B) Expression of GFP and LacZ on day 14 in cultured organoids from *Hopx*$^{LacZ/+}$;*Lgr5*$^{EGFP-ERCre/+}$ mice. LacZ-negative organoids express GFP. **(C and D)** Dual staining for GFP and LacZ of crypt organoids from *Hopx*$^{LacZ/+}$;*Lgr5*$^{EGFP-ERCre/+}$ mice at 1 (C) and 1.5 (D) days of culture. Arrowheads point to double positive cells. Scale bars: 50 μm (A and B) and 20 μm (C and D).

Supporting Figure 6



Supporting Figure 6: Laser capture microdissection (LCM) of *Hopx* descendants **(A)** Cells were captured by LCM at the +4 position 18 hours after tamoxifen pulse or at the crypt base 48 hours after a single tamoxifen pulse from X-gal stained, formalin-fixed, paraffin-embedded sections of $Hopx^{ERCre/+}$;$R26^{LacZ/+}$ intestine, and RNA was extracted (500-700 LacZ-labeled cells/sample). **(B)** Expression levels were normalized to *GAPDH* and expressed relative to levels in CBC cells. Scale bar: 10 μm.

Supporting Figure 7



Supporting Figure 7: Bmi1 expression in *Hopx*$^{LacZ/+}$ mice
(A-C) LacZ and Bmi1 double staining on frozen sections from *Hopx*$^{LacZ/+}$ mice. Arrowheads point to double positive cells. Lower panels in (A) and (B) show high magnification images of double positive cells. **(D)** Bmi1 staining is specifically inhibited with a Bmi1 epitope blocking peptide. Scale bars: 10 μm.